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Two Kinds of Active Factor in Crab Hatch Water: Ovigerous-Hair Stripping Substance (OHSS) and a Proteinase

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Abstract. The embryos of intertidal and estuarine crabs are clustered on the ovigerous seta of the female, where they are ventilated for 2–4 weeks by the female's abdomen. When the embryonic development is complete, hatching occurs and zoea larvae are released into the water. This study indicates that the crab hatch water (*i.e.*, the filtered medium into which zoeas were released) contains at least two kinds of active substance: OHSS (ovigerous-hair stripping substance) and a proteolytic enzyme. Both factors were separated by gel filtration. Powdered fragments of egg capsule were digested by proteinase, suggesting that this enzyme actually acts on the egg capsule. But this activity was at a very low level compared with casein digestion. The proteinase might be digesting the thin, sticky layer enclosing the embryo and would not act on the thick, tough layer constituting the main component of the egg capsule. Therefore, a proteolysis of such low activity could not be expected to cause the egg capsule to rupture.

Introduction

Embryos of intertidal and estuarine crabs are clustered on the ovigerous setae of the female and incubated there for 2–4 weeks. When the embryonic development is complete, hatching occurs and zoeas are liberated into the water by the special fanning behavior of the female (Saigusa, 1982). The timing of hatching is controlled by the circatidal clocks of both the female and the embryos (Saigusa, 1992a, b, 1993), but the physiological mechanism underlying the timing of hatching is not known. To settle this problem, the process of hatching should be studied.

A number of investigations have indicated that the embryos secrete a proteinase upon hatching (for reviews, see Davis, 1981; Yamagami, 1988). These proteinases are thought to dissolve a portion of the egg capsule, thus rupturing it. Some of these proteinases have been purified and characterized: *e.g.*, in the sea urchin blastula (Barrett and Edwards, 1976; Lepage and Gache, 1989; Roe and Lennarz, 1990), fishes (Yamagami, 1972; Hagenmeier, 1974; Yasumasu *et al.*, 1989a, b), and amphibians (Carroll and Hedrick, 1974; Katagiri, 1975).

On the other hand, little is known about the hatching mechanism in Crustacea. Hatching of crustaceans usually occurs upon the rupture of the egg capsule. Many investigations have suggested that this is due to an increase of internal pressure caused by osmotic effects (Yonge, 1937, 1946; Burkenroad, 1947; Marshall and Orr, 1954; Davis, 1959, 1964; Anderson and Rossiter, 1969), or to the swelling of the embryos (Saigusa, 1992b).

Recently, De Vries and Forward (1991) reported that the embryos of a few estuarine crabs release a proteinase upon hatching. But in other experiments, the egg capsules after hatching showed no sign of dissolution (Saigusa, 1992b). So the role of such a proteinase in the hatching of decapod crustaceans remains obscure.

A further question is related to another active factor found in crab hatch water. Hatch water (*i.e.*, the filtered medium into which zoeas have been released) contains an active substance that causes each ovigerous hair to slip out of the investment coat that binds it to the embryo through the funiculus. The embryos is thus lost without

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hatching (for details, see Saigusa, 1994, 1995). A question is whether this factor, which I call OHSS (ovigerous-hair stripping substance), is the proteinase.

I also report here that crab hatch water contains a proteinase. With gel filtration chromatography, the proteolytic activity is eluted in fractions different from those of OHSS. The proteinase certainly dissolves the debris of the egg capsule, but the activity is very weak. Consequently I have concluded that this proteinase does not dissolve the main components of the egg capsule.

Materials and Methods

Hatch water collection

Ovigerous females of *Sesarma haematocheir* that were expected to hatch within a few days were collected from the field at Kasaoka, Okayama Prefecture, and brought to the laboratory. They were dipped into 50% EtOH for a few minutes, for disinfection, and were individually put into beakers (8.5 cm in diameter, 12 cm in height) containing 30 ml of distilled water.

Hatching of estuarine crabs is highly synchronized; in *S. haematocheir*, all zoeas hatched within 5–30 min. As soon as hatching had been completed and the female had released all her zoea larvae into the medium, she was removed, and the medium was filtered through nylon mesh to remove larvae. This filtered medium (*i.e.*, hatch water) was accumulated in the plastic bottles and centrifuged at 15,000 rpm for 30 min to remove solid materials. The hatch water was then lyophilized, and the powder was suspended in 10 m*M* Tris-HCl buffer (pH 8.5). These sample solutions (concentrated hatch water) were centrifuged at 15,000 rpm for 60 min, and were stored at −20°C until used.

Gel filtration chromatography

The sample solutions were applied to a column of Sephacryl S-200 (1.3 × 45 cm) previously equilibrated with 10 m*M* Tris-HCl buffer (pH 8.5). The flow rate was 5 ml per hour, and elution of the proteins was calibrated with blue dextran and NaCl. The proteins contained in each fraction was measured at 280 nm. These experiments were all carried out at 4°C.

Casein assay

Proteolytic activity was assayed with casein (Zwilling and Neurath, 1981). The substrate was 1% casein (Ishizu Seiyaku Co., Japan) that was suspended in 100 m*M* Tris-HCl buffer (pH 8.5) and heated for 10–15 min in a boiling water bath. The assay mixture contained 0.2 ml of 100 m*M* Tris-HCl buffer (pH 8.5), 0.4 ml of 1% casein solution, and 0.2 ml of the enzyme solution. This mixture was incubated for 30 min at 30°C, and was precipitated through the addition of 1.2 ml of 5% TCA (trichloroacetic acid). In control experiments, TCA was added to the 1% casein solution before the enzyme solution. The incubation mixture was then centrifuged at 10,000 rpm for 20 min, and the absorbance of the deproteinized supernatent was measured at 280 nm.

Assay with debris of the isolated egg capsule

Clusters of premature embryos (identified by their brown color; *e.g.*, see Saigusa, 1993, 1994) were detached from the females and folded into a sheet of nylon mesh. They were crushed and washed repeatedly with tap water to remove any embryonic tissue and yolk remaining inside of the egg capsule. The coarse fragments of the egg capsule remaining on the mesh were suspended in distilled water for one night at 4°C. Broken ovigerous setae at the bottom of the glass beaker were removed. After lyophilization, the dried samples were further crushed in a mortar and stored at −20°C.

The powder of the egg capsule debris (30 mg) was suspended in 20 ml of 100 m*M* Tris-HCl buffer (pH 8.5), and this suspension was used as a substrate in the assay of proteolytic activity. The assay mixture contained 0.1 ml of Tris-HCl buffer, 0.2 ml of the substrate, and 0.1 ml of the enzyme solution. This mixture was incubated for 1.5 h at 30°C and precipitated by the addition of 5% TCA (0.6 ml). In the control experiment, 5% TCA was added to the substrate before the enzyme solution. These mixtures were centrifuged at 10,000 rpm for 30 min, and the supernatant was measured at 270 nm.

Assay of OHSS

The activity of OHSS was assayed on clusters of embryos detached from ovigerous females. The ovigerous seta with their premature embryos were separated and cut into 4–6 pieces. Each cluster of embryos was placed in a well of a plastic culture dish and incubated with 0.4 ml of the solution that had eluted upon gel filtration chromatography. After 2 h of incubation at about 25°C, the embryos were transferred to a glass dish with a small quantity of distilled water, then gently pulled with a fine forceps under the stereomicroscope.

The ratio of the number of ovigerous hairs that slipped from the investment coat without breaking, to the total number of hairs on the seta was estimated for each cluster of embryos. The activity of OHSS was determined with respect to the 45% response level (ED_{50}): *i.e.*, the value at which the dose-response curve intersects the de-

fined 45% line (for further details of the assay, see Saigusa, 1995).

Results

Elution of proteolytic activity

Concentrated hatch water (1.5 ml; about five females) was fractionated on the Sephacryl S-200 column with 10 m*M* Tris-HCl buffer, and the proteolytic activity of each fraction was examined. As shown in Figure 1, the proteolytic activity eluted near the void volume of the column. Fractions in the latter half showed no activity.

The molecular size of this proteinase should be determined with gel filtration chromatography. But this active factor could be a product of the enzyme-substrate reaction, since hatch water is the medium into which the zocas were hatched and released. The proteinase could be combining with substrate in the egg capsule (or on the surface of the embryos) to form conjugated proteins. So further investigations will be required to determine the molecular size of this enzyme.

Hatch water was also collected from other species (*S. pictum*, *S. dehaani*, and *Hemigrapsus sanguineus*), subjected to the same gel filtration protocol as was used with *S. haematocheir*, and assayed for proteolytic activity in the same way. Proteolytic activities were detected in the hatch water from all of the species, suggesting that the

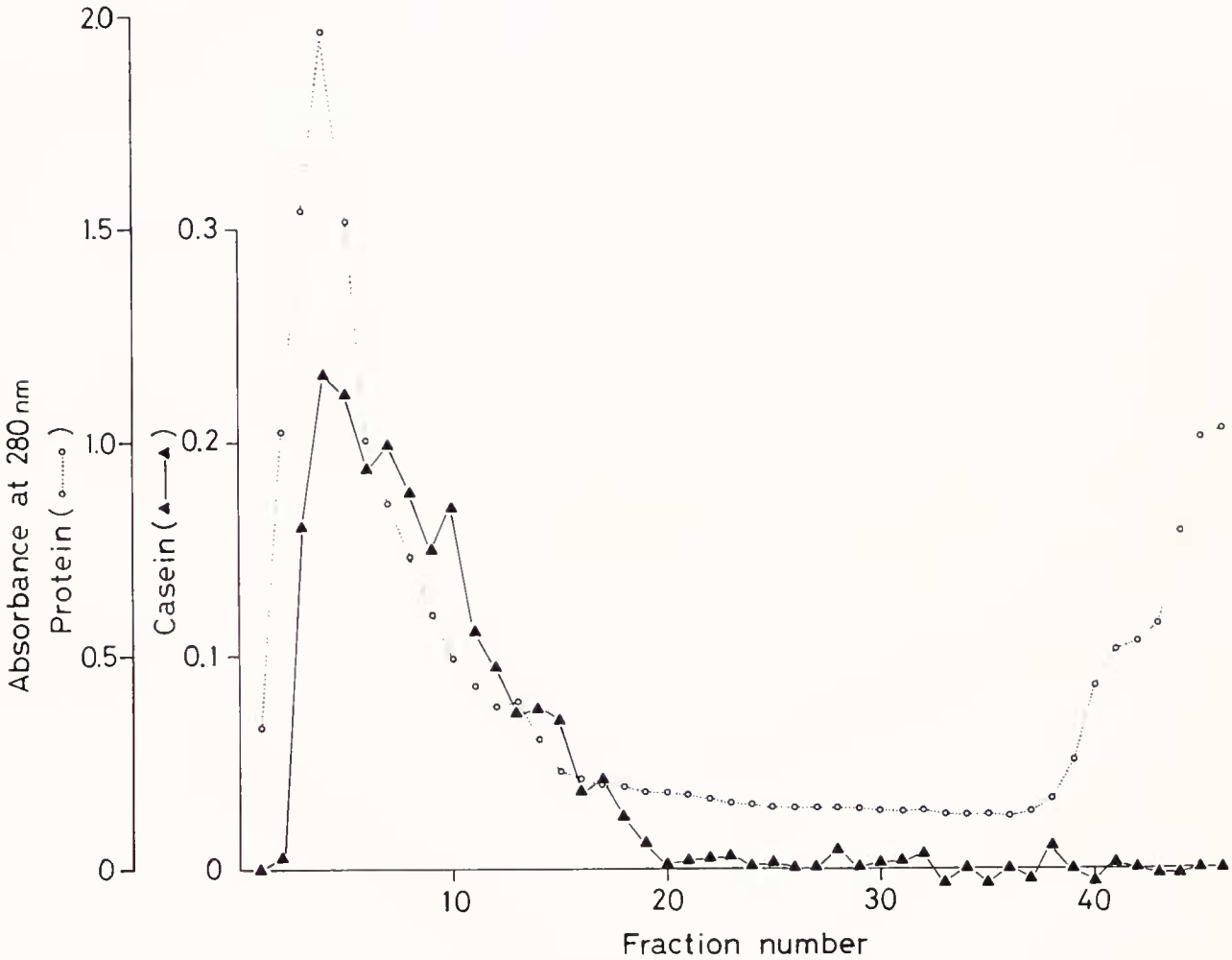

Figure 1. Elution pattern of the proteolytic activity on gel filtration chromatography. A 1.5 ml volume of concentrated hatch water (about 5 females) was adjusted to a Sephacryl S-200 column previously equilibrated with 10 m*M* Tris-HCl buffer (pH 8.5). Casein solutions (1%) were incubated for 30 min at 30°C with each fraction, and the absorbance of the deproteinized supernatant was measured at 280 nm (○·····○). Absorbance of protein (▲ — ▲).

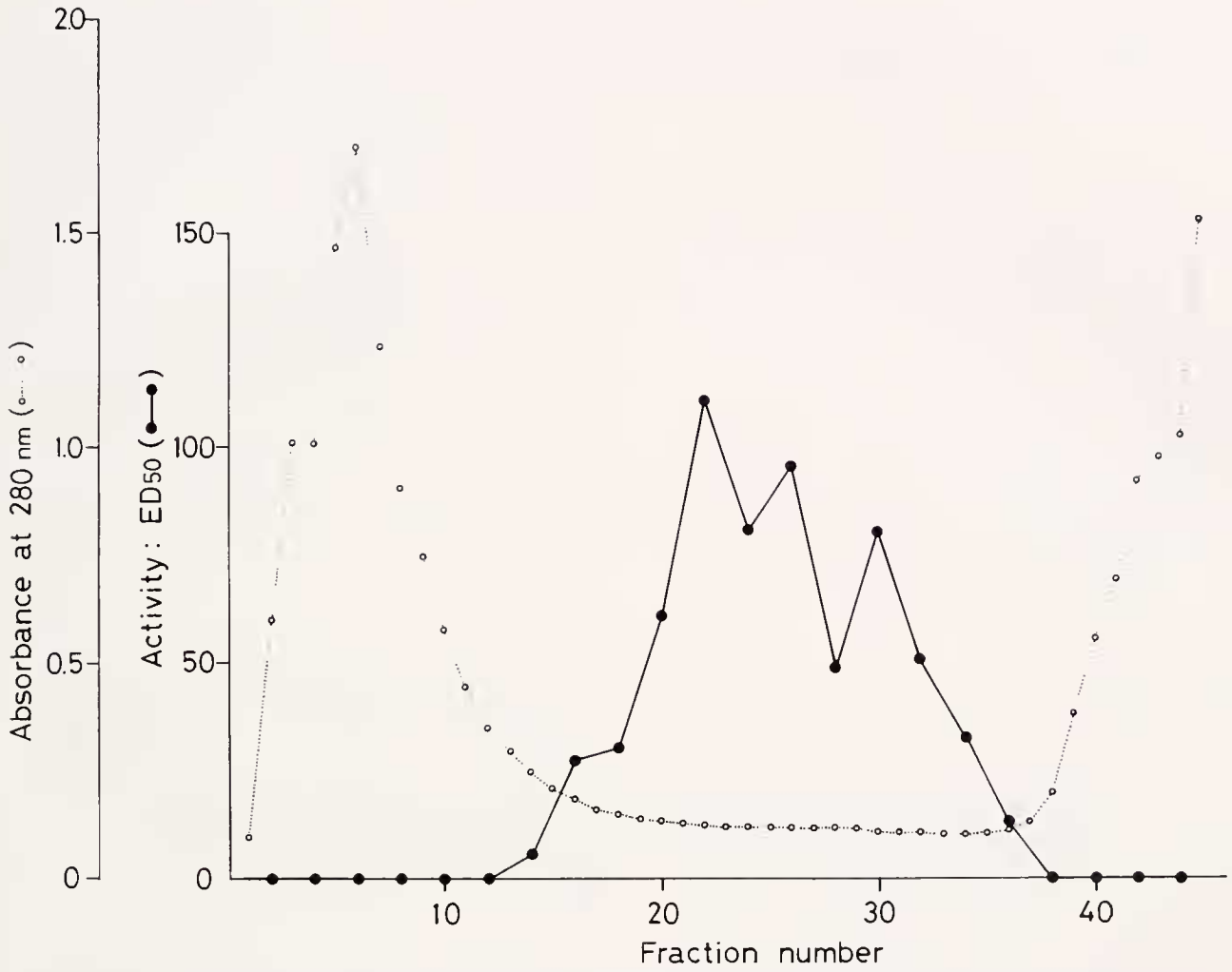

Figure 2. Elution pattern of OHSS activity on gel filtration chromatography. Concentrated hatch water (1.5 ml; 5 females) was subjected to the same gel filtration protocol shown in Figure 1. Clusters of embryos, freshly detached from three females, were incubated with each fraction for 2 h at about 25°C. OHSS activity (● — ●); Absorbance of protein (○·····○).

proteinase occurs widely in intertidal and estuarine crabs (data not shown).

Elution of OHSS activity

Concentrated hatch water (1.5 ml; about five females) was subjected to the same gel filtration protocol used in the experiment of Figure 1. The activity of each fraction was assayed with unhatched embryos of *Sesarma haematocheir* for 2 hours (Fig. 2).The activity of OHSS extends over a wide range of fractions. As shown elsewhere (Saigusa, 1995), the molecular size of OHSS was estimated to be 15–20 kDa by a comparison of its elution volume with those of the standard proteins.

The pattern of OHSS activity on gel filtration (Fig. 2) is clearly different from that of proteinase (Fig. 1). Therefore, at least two kinds of active factors are contained in crab hatch water, and both factors occur widely in intertidal and estuarine crabs.

Assay with egg-capsule debris

I next asked whether the hatch water would dissolve the egg capsule, and if so, whether OHSS and proteinase could be responsible for the activity. The assay was therefore performed with the powdered debris of the egg capsule as substrate. Hatch water (1.5 ml; about five females) that had been lyophilized and suspended in 10 m*M* Tris-HCl buffer (pH 8.5) was subjected to the same gel filtration protocol used in the experiments shown in Figures 1 and 2.

The debris of the egg capsule was dissolved by the

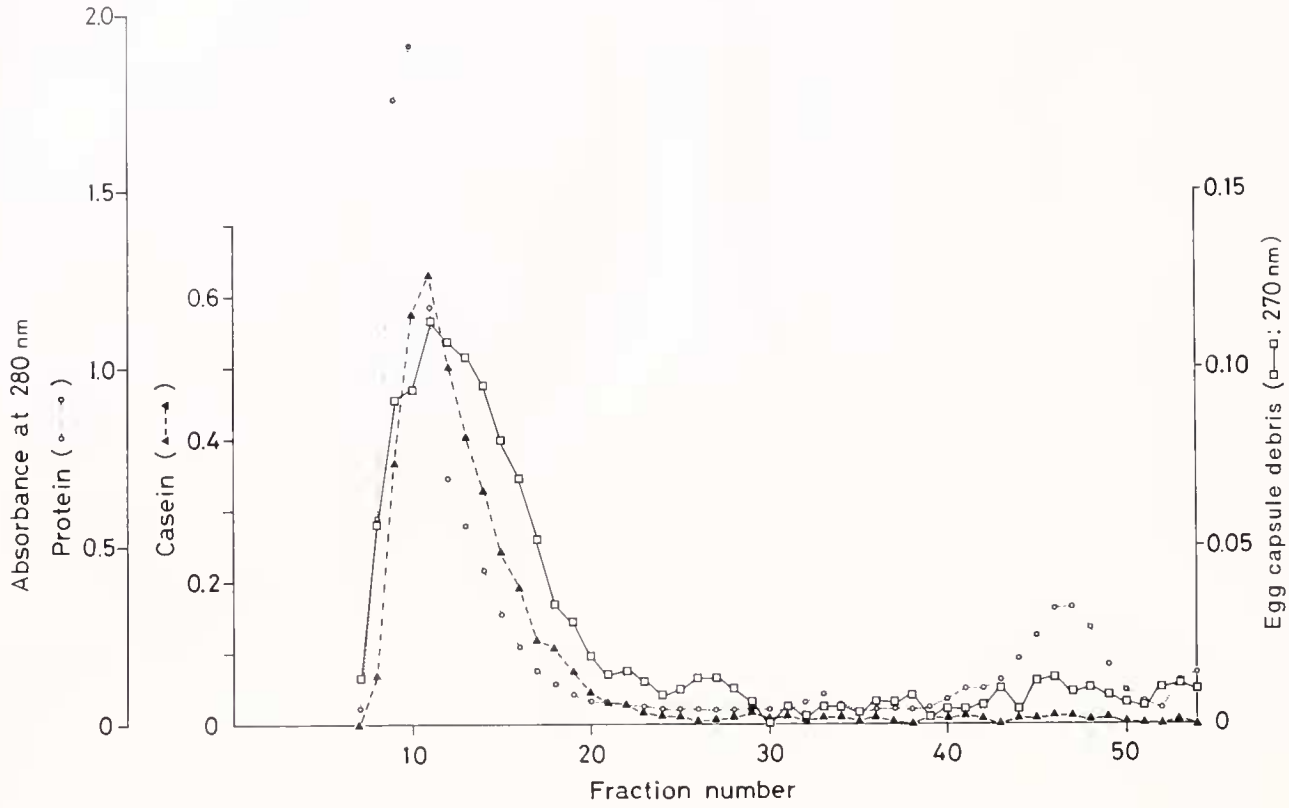

Figure 3. Dissolution of egg capsule debris by the proteinase. Concentrated hatch water (1.5 ml; 5 females) was subjected to the same gel filtration chromatography shown in Figure 1. The substrate solutions containing egg capsule debris or 1% casein were assayed with each fraction. Both solutions were incubated for 1.5 h at 30°C. Absorbance of protein (○ ····· ○); proteolytic activity (▲- - -▲); digestion of egg-capsule debris (□ — □).

hatch water (Fig. 3). The activity eluted as a single peak, and its pattern clearly agrees with that of the proteolytic activity (Fig. 1), and not with OHSS (Fig. 2). Thus the egg capsule debris is probably digested by the proteinase, but not by OHSS.

Some other experiments

To determine whether a chitinase is contained in the hatch water, degradation of colloidal chitin (*i.e.*, a homopolymer of β-linked *N*-acetylglucosamine) was examined. The assay method followed was that of Bade and Stinson (1979). In addition, the release of *p*-nitrophenol from 0.1 m*M* *p*-nitrophenyl 2-acetoamide-2-deoxy-β-D-glucopyranoside (*p*NP-β-GlcNAc) solution. The assay method was that of Dziadik-Turner *et al.* (1981). Neither *N*-acetylglucosamine nor *p*-nitrophenol was released from these substrates, suggesting that a chitinase is not contained in crab hatch water (data not shown).

Proteolytic activity was also assayed with an amidase substrate, *i.e.*, *N*-benzoyl-L-valylglycyl-L-arginine-*p*-nitroanilide (BValGlyArgNA); the method was that of Grant *et al.* (1981). BValGlyArgNA was dissolved in 50 m*M* Tris-HCl buffer (pH 8.5) to a concentration of 1 mg/ml (1.7 m*M*). The solution was further diluted 20 times with 100 m*M* Tris-HCl buffer (pH 8.5). The assay mixture contained 200 μl of this diluted substrate solution and 200 μl of the enzyme solution. The amount of *p*-nitroanilide liberated was monitored continuously at 385 nm for 4–5 min. Concentrated hatch water (1.5 ml; five females) was subjected to the same gel filtration protocol used in Figures 1 and 2.

This substrate (*i.e.*, BValGlyArgNA) was also decomposed by hatch water. The pattern of this activity on gel filtration was the same as that shown in Figure 2, suggesting that BValGlyArgNA is decomposed by the proteinase, not by OHSS (data not shown).

Discussion

This study indicates that the hatch water of the estuarine terrestrial crab *Sesarma haematocheir* contains a

proteolytic enzyme (Fig. 1) in addition to OHSS (*i.e.*, ovigerous-hair stripping substance) (Fig. 2). Both factors were separated by gel filtration. The proteinase dissolved the egg capsule debris, but the absorbance at 270 nm was very low, even at its peak, compared with the dissolution of casein (Fig. 3). This result raises a question about the function of the proteolytic activity: what part of the egg capsule is dissolved by this enzyme?

The embryos of crustaceans are encased in capsules comprising at least two distinct layers as observed with the stereomicroscope, although they may consist of several layers when examined at higher magnifications (Cheung, 1966; Goudeau and Lachaise, 1980). But the principal components are the outer layer, which is thick and tough, and the inner layer, which is very thin (Yonge, 1937, 1946; Marshall and Orr, 1954; Davis, 1959, 1964, 1965; and Anderson and Rossiter, 1969). A number of studies have suggested that the outer thick layer is not dissolved, but that it cracks when hatching occurs (for a review, see Davis, 1981). Nevertheless, a proteinase is released outside of the egg capsule at the time of hatching (Fig. 2), and it dissolves the debris of the egg capsule (Fig. 3). So where does the proteinase act?

In addition to these two layers, embryos of decapod crustaceans are invested by a transparent layer consisting of very sticky material. The existence of this layer becomes clear at the time of hatching, when the zoeas try to leave the broken egg capsule. This structure protrudes from the broken egg case upon the escape of the zoea (see fig. 3C in Saigusa, 1993). The sticky nature of this material is apparent when it is picked out with a forceps. The hatched zoeas escape from this layer by vigorous vibrating movements of their abdomen and limbs. Observations with a scanning electron microscope show this layer to be very thin and irregular in structure (see fig. 5b in Saigusa, 1992b).

For the experiment shown in Figure 3, the isolated egg capsules were washed repeatedly with tap water until the yolk was completely gone. Nevertheless, a small quantity of the fragments of embryonic tissues might have remained. Although the notion that those fragments is digested still remains, I suppose that the proteinase hydrolyzes this sticky layer upon hatching. This layer is very thin in the egg capsule, so the absorbance at 270 nm would have been very low. Yet, it is doubtful that such a small-scale dissolution of the egg capsule (Fig. 3) directly causes a rupture of the tough egg capsule of crustaceans.

Acknowledgments

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